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ASCORBIC ACID FOLIIAR SPRAY COUNTERACTING EFFECT OF SALINITY ON GROWTH, NUTRIENTS CONCENTRATIONS, PHOTOSYNTHESIS, ANTIOXIDANT ACTIVITIES AND LIPID PEROXIDATION OF BEAN (*PHASEULUS VULGARIS* L.) CULTIVARS

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ABSTRACT

A water culture experiment was carried out in the Department of Fertilization Technology at National Research Centre, Cairo, Egypt, to investigate the effect of Ascorbic Acid (AsA) foliar application and salinity stress (100 ppm and 100 mmoL NaCl) on growth, nutrients concentration and some biochemical parameters of two kidney bean (*Phaseulus Vulgaris* L.) cultivars. Salinity caused significant reduction in growth parameters (leaves and root dry weights) and some of biochemical parameters (nutrients concentration, photosynthesis pigment, Carbonic Anhydrase Activity (CAA), antioxidant enzyme activity Peroxidase (POD) and lipid peroxidation). The reduction effect on Paulista cultivar was higher than Nebraska cultivar at 100 mM NaCl salinity stress. Meanwhile, POD activity was increased under salt stress conditions. Lipid Peroxidation (LP) under 100 mmoL NaCl salinity was significantly increased. The two cultivars showed an increase in MDA content with NaCl salinity stress, but the increase in sensitive cultivars Paulista was higher than that in salt-tolerant Nebraska cultivar. Application of (AsA) not only mitigated the inhibitory effect of salt stress in both kidney bean cultivars, but also in some cases induced a stimulatory effect greater than that estimated in the control plants on growth parameters which were accompanied by marked increases in nutrients concentration and photosynthetic system (pigments and carbonic anhydrase activity). Ascorbic Acid (AsA) has been shown to be an essential antioxidant; agent may act as a scavenger of ROS for mitigating the injury on bio-membranes under salt stress. Therefore, this study suggested that (AsA) application may induce an adaptive response in kidney bean through stimulation of the antioxidant enzymes activities, photosynthesis processes and lower lipid peroxidation, in Nebraska relative to Paulista may contribute to salt tolerance mechanism in Nebraska.

Keywords: Salinity Strss, Antioxidants, Ascorbic Acid, Kidney Bean

1. INTRODUCTION

Salinity is one of the most important abiotic stresses which affect many aspects of plants metabolism and reduce growth and crop production (Zhu, 2002). When plants are subjected to environmental stresses, Reactive Oxygen Species (ROS) are generated in response to stress condition (Dat *et al*., 2000). Salinity causes numerous physiological and biochemical changes in plants like reduced leaf size, stem extension, root proliferation, reduced water use efficiency (Farooq *et al*., 2009) Alteration in metabolic activities (El-Fouly and Salama, 1999), inhibition

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of enzymatic activities (Frova *et al*., 1999) ionic imbalance and disturbances in solute accumulation (El-Fouly *et al*., 2010) or a combination of all these factors. Excess of ROS causes phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation (Wang *et al*., 2003).

Ascorbic acid is a natural product of plants functions play a key role as an antioxidant and an enzyme and apparently plays a role in ameliorating cofactor. It participates in a variety of processes. Ascorbic acid is associated with chloroplasts the oxidative stress of photosynthesis. In addition, AsA has a number of other roles in cell division and protein modification. One approach for inducing oxidative stress tolerance would to acts as a primary substrate in the cyclic pathway of enzymatic detoxification of hydrogen peroxide (Beltaji, 2008). Ascorbic acid application was also alleviated the destructive effects of salinity on osmotic potential, shoot and root dry mass, K^+/Na^+ ratio and contents of photosynthetic pigments in wheat seedlings under salinity stress was completely affected by exogenous ascorbic acid (Kaydan *et al*., 2007). There were various attempts to improve the salinity tolerance of a variety of crops by traditional breeding programs. Little information on how ascorbic acid regulates physiological/biochemical processes in kidney bean plants subjected to salt stress. Therefore, this study was to evaluate the counteracting effects of ascorbic acid foliar spray on growth, nutrients concentrations, photosynthesis, antioxidants enzymes and lipid peroxidation were evaluated for two cultivars of kidney bean (*Phaseulus vulgaris* L.) grown hydroponically under salt stress conditions.

2. MATERIALS AND METHODS

2.1. Plant Materials and Growth Conditions

A Water culture experiment was carried out at Fertilization Technology Department, National Research Centre, Cairo, Egypt. Seeds of two cultivars of Kidney Bean *Phaseulus Vulgaris* L. Paulista and Nebraska were obtained from Agricultural Research Institute. The seeds were washed and soaked for several hours in aerated tap water. The germination was carried out in plastic dishes at 28°C in dark. Three days-old seedlings were put to grow in plastic pots filled with one-tenth concentration of Hoagland and Arnon (solution (pH 6.0) containing 5 mM$Ca(NO_3)_2$. $4H_2O$, 5 mM KNO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4.7H_2O$ and micronutrients in µM: H_3BO_3 -0, $MnCl_2$ -0.5, $ZnSO_4$ -0.5, $CuSO_4$ -0.2, Na_2MoO_4 -0.1, Fe (III)-HEDTA -20). The seedlings were grown in an environmental growth chamber under 16 h light at 120 µmoL m-2 sec-1 provided by fluorescent tubes, 8 h night, 60% Relative Humidity (RH) at 25°C day/20°C night temperature. Two days later the plants were divided into four variants (plus and minus NaCl) with and without spraying ascorbic acid (0.00-100 ppm) (50-100 mL) were applied for each pot. It was used to correct the nutrient imbalance caused by salt stress conditions. At 21 days old. Samples were taken for plant growth measurements and determine of nutrient contents according to (Chapman and Pratt, 1978).

2.2. Chemicals

All Chemicals were of the Highest Commercial Grade and Obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.3. Nutrients concentration

Total elements were analyzed after digestion of plant samples with mixture of concentrated HSO_4 and perchloric acids. The total macro and micronutrient concentrations: K, Ca and Mg, Fe, Mn and Zn were analyzed in plant digests using Flame photometer and atomic-absorption spectrophotometry. All macronutrients were expressed in g/100 gdry matter and micronutrient concentrations were expressed in mg/kg DW according to (Chapman and Pratt, 1978).

2.4. Chlorophyll Measurement

Samples (100 mg leaves) were homogenized in chilled 80% (v/v) acetone and centrifuged at 10 000 g for 10 min at 4°C. Absorbance of the acetone extracts was measured at 663 and 645 according to (Lichtenthaler, 1987).

2.5. Enzyme Assays

2.5.1. Preparation of Enzyme Extracts

About 5.0 g were crushed into fine powder using liquid nitrogen. Soluble protein was extracted by homogenizing the powder in 10 mL of 50 mM phosphate buffer (pH 7.8) containing 1mM EDTA and 1% PVP, with the addition of 1 mM ascorbate in the case of POD assay at 4°C. The homogenate was centrifuged at 15,000×g for 20 min and the supernatant was used for the following enzyme activity assay.

2.5.2. Peroxidase Activity (EC1.11.1.7) Assay

Peroxidase activity was assayed by monitoring the increase in absorbance at 430 nm due to the oxidation of pyrogallol (Amako *et al*., 1994). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 20 Mm pyrogallol, 5 mM H_2O_2 and 20 µL of enzyme extract. One unit of enzyme was the amount necessary to

decompose 1 µmoL of substrate per minute at 25°C. Peroxidase activity was expressed as EU/gfw/ min^{-1}.

2.5.3. Carbonic Anhydrase Activity Assay (EC 4.2.1.1)

Leaves tissue (100 mg FW) were placed into liquid nitrogen and then homogenized with a buffered solution (pH 8.3) that contained 50 mm Veronal-H_2SO_4 and 0.2% (w/v) PVP under ice cold-conditions. The homogenate was centrifuged at 12,000 g for 2 min and the supernatant was used for the determination of CA activity according to (Ohki, 1978).

2.5.4. Determination of Lipid Peroxidation (L.P)

Lipid peroxidation was measured as the amount of MDA determined by the Thiobarbituric Acid (TBA) reaction (Heath and Packer, 1968). Frozen samples were homogenized with a pre-chilled mortar and pestle with two volumes of ice-cold 0.1% (w/v) TCA and centrifuged for 15 min at 15 000 g. Assay mixture containing 1 mL of the supernatant and 2 mL of 0.5% (w/v) TBA in 20% (w/v) TCA was heated at 95°ë for 30 min and then rapidly cooled in an ice bath. After centrifugation (10000 g for 10 min at 4°C), the supernatant absorbance was read at 532 nm and the values corresponding to nonspecific absorption (600 nm) were subtracted. Lipid peroxidation products were measured as the content of TBA-reactive substances. The MDA content was calculated according to the molar extension coefficient of 155/(mM cm^{-1}).

2.6. Statistical Analysis

Data were statistically analyzed using Costat statistical package according to (Anonymous, 2000).

3. RESULTS

3.1. Effect of (AsA) Foliar Spray on the Plant Growth of Kidney Bean Cultivars Grown In Salinized Media

Data presented in (**Fig. 1**) indicate the effect of (AsA) foliar spray on growth (leaves and roots) dry matter per plant) of Kidney Bean cultivars grown in salinized media. NaCl Salinity had a negative effect on shoot and root dry weights for Nebraska. At 100 mM NaCl treatment, reduction in dry weight was more in both salt-sensitive and a salt tolerant kidney bean plant when compared with control (**Fig. 1**). Ascorbic acid application alleviated the NaCl toxicity and minimized the reduction in dry weights caused by NaCl. In general, effects of ascorbic acid in mitigating the adverse effects of salt stress have been described to activation of some of the enzymatic reactions. It is evident that ascorbic acid plays a key role in the regulation of a number of metabolic processes in plants exposed to salt stress.

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3.2. Effect of (AsA) Foliar Spray on Nutrients Concentration of Kidney Bean Cultivars Grown in Salinized Media

Concerning nutrients concentration in leaves of bean cultivars, high salt concentration in the growth root medium was found to limit the uptake of all determined nutrients with different degrees. Potassium (K, Ca and Mg) tended to decrease with NaCl level increase. Salinity induced K^+deficiency. Also, reduced Ca and Mg contents in leaves of both cultivars (**Fig. 2**). Reduction of iron concentration was higher in the aerial parts compared to control Unexpected results for Mn concentration it was greatly increased in both cultivars with treatment 100 mmol NaCl, while Zn concentration in the arial parts of both cultivars were slightly decreased compared with control plants. Ascorbic acid spray improved the performance of root growth and prevented the nutritional disorders and consequently caused increases of macronutrients and micronutrients uptake (**Fig. 2**).

3.3. Effect of Ascorbic Acid (AsA) Foliar Spray on Photosynthetic Pigments Content in Leaves of Kidney Bean Cultivars Grown In Saline Media

The effect of salinity in presence or absence of (AsA) on photosynthesis pigments is presented in (**Table 1**). Imposition of salt stress caused a significant reduction in chlorophyll a, b and total chlorophyll (a+b) of the seedlings of both cultivars. Application of 100 ppm ascorbic acid as a foliar spray enhanced chlorophyll a, chlorophyll b and T-Chl. content in both bean cultivars under saline conditions (**Table 1**). The reduction in photosynthetic pigments was significantly in *Nepraska* compared to paulista cultivar. Application of Vit C. did not only alleviate the inhibitory effect of salinity stress on the biosynthesis photosynthetic pigments, but also induced a significant stimulatory effect greater than observed in the control treatment.

Table 1. Effect of ascorbic acid 100 ppm in presence or absence of 100NaCl on Chl.a, Chl.b,T.Chl.(a+b), CAA, POD and L.P grown hydroponically for three weeks

Treatments	Chl.a µg/fw	Chl.b µg/fw	Chl (a+b) µg/fw	CA EU/gfw	POD EU/gfw/min^{-1}	L.P µmol/fw
Paulista						
Control	16.65^{c}	6.21^{c}	22.86^{c}	214^{c}	240^{b}	2.50^{a}
Control+Vit.C	17.17^{d}	6.35^{c}	23.52^{c}	315^{d}	334^{c}	2.70^{b}
100mmolNaCl	11.48^{a}	4.83^{a}	16.31^{a}	164^{a}	421^{d}	4.16^{d}
NaCl+Vit.C	13.33^{b}	5.34^{b}	18.67^{b}	175^{b}	143^{a}	3.12^{c}
LSD at 0.05	0.07	0.42	0.87	6.26	36.23	0.29
Nebraska						
Control	18.53^{b}	7.50^{c}	26.03^{a}	415^{c}	592^{ab}	3.60^{a}
Control+Vit.C	21.29^{c}	8.06^{d}	29.35^{b}	401^{b}	948^{b}	2.80^{b}
100mmolNaCl	9.77^{b}	4.02^{a}	13.79^{c}	328^{a}	950 ^{ab}	3.60^{b}
NaCl+Vit.C	10.37^{a}	4.42^{b}	14.79^{d}	401^{b}	336^{a}	3.80^{b}
LSD at 0.05	0.40	0.28	0.53	10.51	303.32	0.33

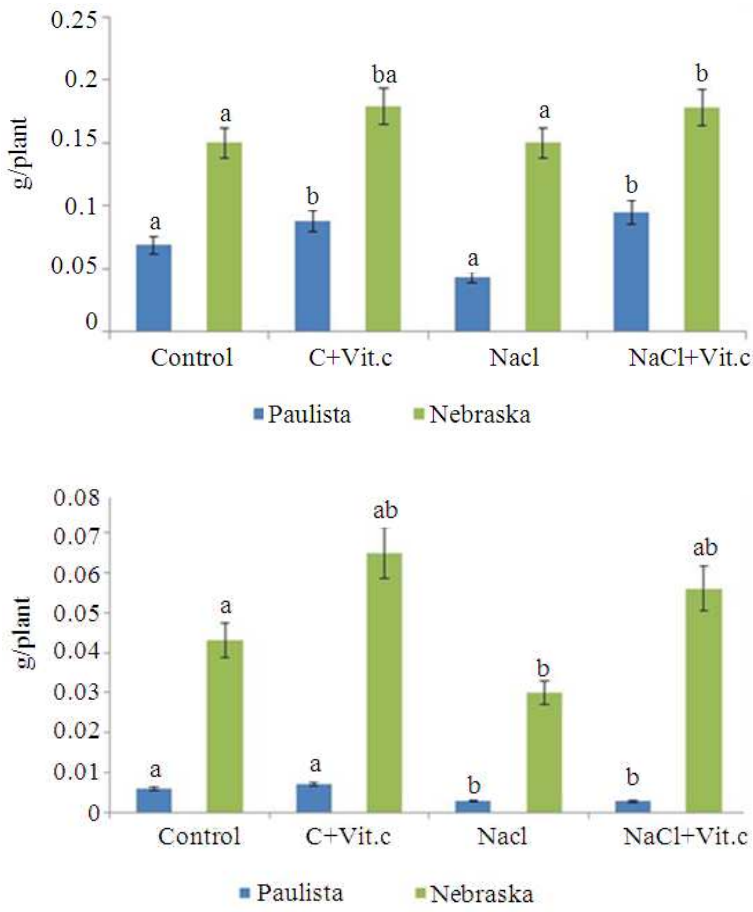
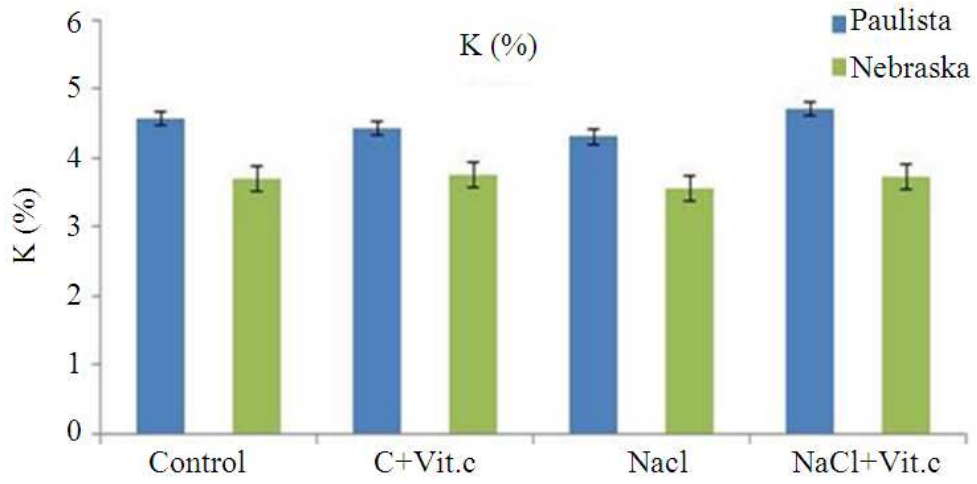
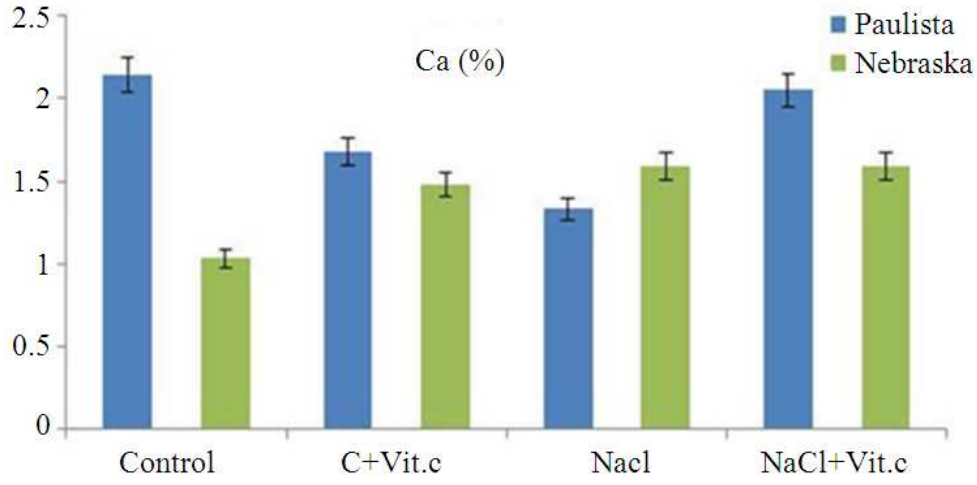
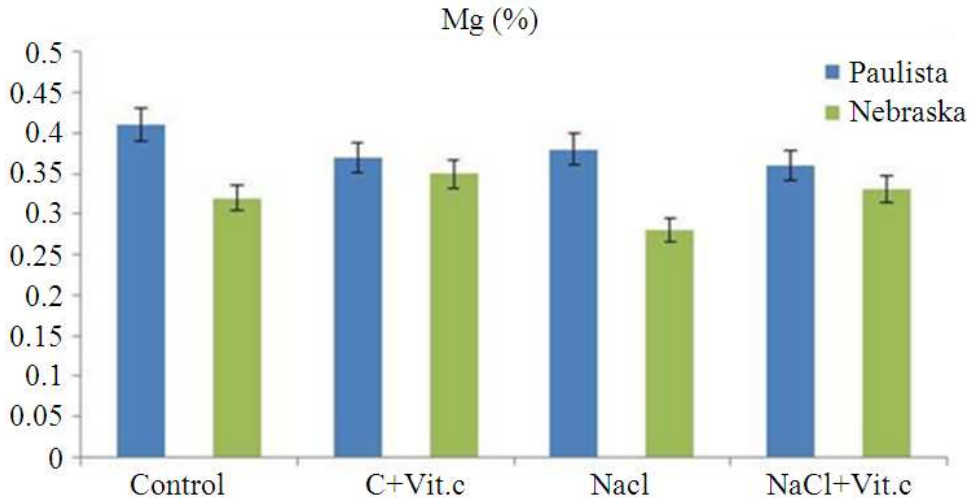


Fig. 1. Effect of ascorbic acid 100 ppm foliar spray in presence or absence of 100 NaCl on leaves and root dry weight (g/plant) of two cultivars of kidney bean grown hydroponically for three weeks


K (%)
Paulista
Nebraska
6
5
4
3
2
1
0
K (%)
Control
C+Vit.c
Nacl
NaCl+Vit.c


Ca (%)
Paulista
Nebraska
2.5
2
1.5
1
0.5
0
Control
C+Vit.c
Nacl
NaCl+Vit.c


Mg (%)
Paulista
Nebraska
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
C+Vit.c
Nacl
NaCl+Vit.c

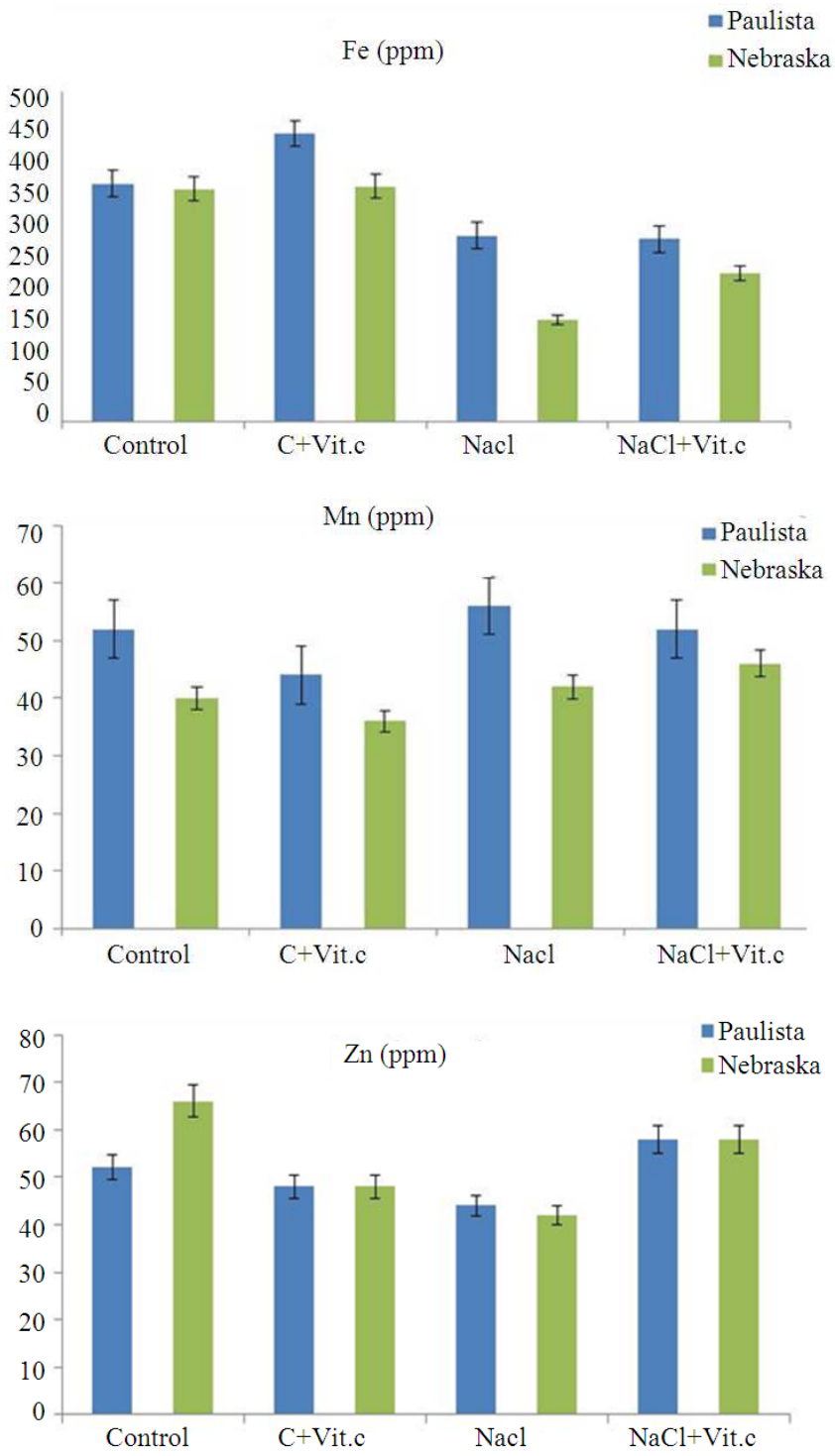


Fig. 2. Effect of ascorbic acid 100 ppm in presence or absence of 100 NaCl on K,Ca,Mg, Fe, Mn and Zn concentration of two cultivars kidney bean grown hydroponically for three weeks

3.4. Effect of Ascorbic Acid (AsA) Foliar Spray on Carbonic Anhydrase Activity (CAA) in Leaves of Kidney Bean Grown in Saline Media

The effect of NaCl on CAA for two cultivars of bean was presented in (**Table 1**). The activity of CA was significantly decreased by the NaCl treatment (**Table 1**). The photosynthetic carbon assimilation process in bean cultivars was found to be significantly depressed by 100 mmoL NaCl. The inhibition of CAA after adding 100 mmoL NaCl was (23.4 and 21%) for *Paulista* and *Nebraska* with respect to control. There was an unexpected decline on CAA activity when 100 ppm (AsA) was applied for non saline conditions. Ascorbic acid treatment improved the activities of CA in Paulista plants that were subjected to salinity stress. While no change in the activities of CA in Nebraska plants receiving both NaCl (100 mM) and AsA.

3.5. Effect of Ascorbic Acid (AsA) Foliar Spray on Antioxidant Enzyme Activity (POD) in Leaves of Kidney Bean Cultivars Grown in Saline Media

A considerable increase in the peroxidase activity in leaves of kidney bean cultivars grown with NaCl 100 mmoL are shown in (**Table 1**). The highest enzyme POD activity of plants maintained at 100 mmoL NaCl level was 421 and 950 ΔA/gfw min-1 for Paulisita and Nebraska respectively. The POD activity decreased to 143 and 336 ΔA/gfw min-1 for Paulista and Nebraska after (AsA) application. POD was higher in salt-tolerant Nebraska than in salt-sensitive Paulista at 100 mmoL NaCl treatment.

3.6. Effect of (AsA) Foliar Spray on Lipid Peroxidation of Kidney Bean Cultivars Grown in Salinized Media

To evaluate the NaCl-induced oxidative damage to the cell membranes, the content of Thiobarbituric Acid Reactive Substances (TBARS) was determined. Lipid peroxidation in leaves of both kidney bean cultivars, measured as MDA content, is given in (**Table 1**). NaCl treatments led to an increase in the levels of MDA in both cultivars. Data presented in (**Table 1**) showed that (LP) gave its higher value (4.16 and 3.60 μmol.gfw^{-1}) when Paulista and Nebraska grown with NaCl 100mmol compared to control (2.5 and 3.6 umol.gfw^{-1}. Accumulation of MDA in Paulista, however, was higher than in Nebraska, indicating a higher degree of lipid peroxidation due to salt stress. Nebraska showing better growth, had less MDA and under salt stress had less MDA than Paulista, exhibited less active lipid peroxidation, indicating that Nebraska was the salt-tolerant cultivar. This indicates that salt-tolerant cultivar induced capability of plant protection against oxidative damage caused by salt treatment.

4. DISCUSSION

The present study explore the countracting effect of (AsA) on two cultivars of kidney bean paulista and nebraska under salinity stress conditions. Under saline conditions results of our investigation showed that salt stress reduced plant growth for both cultivars.

It has been reported that the typical symptom of salinity injury to the plant is growth retardation due to the inhibition of cell elongation (Fariduddin *et al*., 2003). In this study, when subjected to 100 mmoL NaCl, both cultivars Paulista and Nebraska. Showed a reduction in plant growth. The response of two kidney bean cultivars to salt stress was found to be different. Nebraska showed better growth than Paulista under salt stress conditions.

Reduction of plant growth by salinity has been reported in several grain legumes, including *Phaseolus vulgaris*, the decrease of leaves and roots dry weight may be attributed to NaCl altering the water potential, increase ion toxicity or causing ion imbalance (Al-Ansari, 2003; Morant-Manceau *et al*., 2004). Such depressive effect of salinity in peas growth may be also, attributed to the adverse effect on enzymatic processes through some interactions of salts and some organic substances of the cell (Oertil, 1996). In general, effects of ascorbic acid in mitigating the adverse effects of salt stress have been described to activation of some of the enzymatic reactions. It is evident that ascorbic acid plays a key role in the regulation of a number of metabolic processes in plants exposed to salt stress.

High salt concentration in the growth root medium was found to limit the uptake of all determined nutrients with different degrees. Under salt stress Salinity induced K^+deficiency which has been implicated in growth and yield reduction of various crops, spinach (Chow *et al*., 1990) and maize (Botella *et al*., 1997). The increment of Ca concentration in the leaves of *Nebraska* cultivar may be due to that most of Ca migrates to the shoot as an osmoregulation to resist salt determined effects on shoot cells. Reduction of iron concentration was higher in the aerial parts compared to control which suggest that Fe accumulates in the roots because of lack of the processes energize translocation and a pH effect (Carvajal *et al*., 1999). These findings are also agree with results obtained by (Achakzai, 2008) in sorghum and maize seedlings subjected to various levels of water stress conditions, as well as in uptake and accumulation of

macronutrients by wheat as reported by (Achakzai, 2008). Ascorbic acid application (100 ppm) increased the concentration of K, Ca, Mg, Fe, Mn and Zn. Ascorbic acid spray improved the performance of root growth and prevented the nutritional disorders and consequently caused increases the uptake of nutrients (El-Fouly and Salama, 1999; El-Fouly *et al*., 2002; 2010). It has been found that ascorbic acid strongly inhibited Na^+ and Cl^-accumulation of salt stressed maize plants, but stimulated N, Mg, Fe, Mn and Cu concentrations. These results suggest that ascorbic acid could be used as a potential growth regulator to improve plant salinity stress resistance (Gunes *et al*., 2007).

The decrease of photosynthesis pigments under salt stress due to stomata closure, inhibition of chlorophyll synthesis, a decrease of carboxylase due to high chlorophylase activity (Batanouny *et al*., 1991). Also, (Agastian *et al*., 2000) reported that the changes in leaf chlorophyll content may have been due to reduced biosynthesis or increased degradation of chlorophyll under saline conditions. Furthermore, (Mittler, 2002) suggested that in salt stressed plants, breakdown of ultrastructure of chloroplasts including plastid envelop, thylakoids and photosynthetic apparatus may result due to direct Na^+ toxicity or salt-induced oxidative damage. Adverse effect of salt stress on chlorophyll '*a*' was counteracted by (AsA) application. Smirnoff (1993) stated that ascorbate has a central role in photosynthesis in chloroplast and protected photosynthetic apparatus from salt induced oxidative stress. Ascorbic acid treatment also increased the level of chlorophyll in the present investigation. Which is well supported by the earlier observations in wheat and/or mung bean seedlings under stress free conditions (Moharekar *et al*., 2003; Hayat *et al*., 2005) as well as under water stress (Singh and Usha, 2003).

Salt stress is reported to damage the photosynthetic machinery at multiple levels, such as pigments, stomatal functioning and gaseous exchange, structure and function of thylakoid membrane, electron transport and the sensitivity of CAA to chloride ions (Sudhir and Murthy, 2004). Excess salt concentration cause the closure of stomata, by decreasing the partial CO_2 pressure (Bethkey and Drew, 1992) as well as internal CO_2 concentration and consequently the activity of carbonic anhydrase (**Table 1**). There was an unexpected decline on CAA activity when 100 ppm (AsA) was applied none saline conditions because its activity is to large extent regulated by the CO_2 concentration (Tiwari *et al*., 2005). Therefore, the level of CAA in treated plants with (AsA) was lower than those which did not receive (AsA) treatment due to increased stomatal conductance as well as the internal CO_2 concentration in stress-free plants (Fariduddin *et al*., 2003). Ascorbic acid treatment also improved the activities of CA in the plants that were subjected to salinity stress. The activities of enzyme CA in the plants receiving both NaCl (100 mM) and AsA were 6.7 and 22.30% higher than those receiving NaCl (100 mM) alone. Ascobicacid (AsA) plays a key role in the activation of rubisco and PEP carboxylase and CA under stress as documented by explain the (AsA) mediated elevation in the activity of CAA is that it corrects the stress mediated damage to the plasma membrane, as evident from an increase in the membrane stability (Khan *et al*., 2003).

A considerable increase in the peroxidase activity POD of paulista and Nebraska cultivars at 100 mmoL NaCl treatment. Over expression of the POD gene in plant has been reported to improve protection against oxidative stress (Wang *et al*., 2003). These results are consistent with observations of many researchers who reported that POD activity plays a central protective roleduring salts tress (Neto *et al*., 2006). These enzymes were also reported to be important insalt tolerance in mulberry (Sudhakar *et al*., 2001) and maize genotypes (Neto *et al*., 2006).

NaCl treatments led to a significant increase in the content of MDA in both kidney bean cultivars. Nebraska cultivar showing better growth under salt stress had less MDA than Paulista indicating that salt-tolerant Nebraska exhibited less active lipid peroxidation under 100 mmoL NaCl salinity treatment than the salt-sensitive Paulista. This indicates that salt-tolerant cultivar induced capability of plant protection against oxidative damage caused by salt treatment. NaCl application was stimulatory for MDA acumilation which almost higher in Paulista. Lipid Peroxidation (LP) is associated with damages provoked by a variety of environmental stresses. Poly-Unsaturated Fatty Acids (PUFA) are the main membrane lipid components susceptible to peroxidation and degradation (Elkahoui *et al*., 2005). The increase in LP, as caused by NaCl stress in the present study, can be correlated to ion accumulation and AOS production under salt stress (Hernandez *et al*., 2000). The LP level indicates the extent of salt tolerance in the given cultivars (Bor *et al*., 2003; Neto *et al*., 2006).

5. CONCLUSION

Our results indicate that, even if oxidative stress is induced in kidney bean plants grown under NaCl salt stress reduced all growth parameters, nutrients uptake,

photosynthesis mechanism, antioxidant system which involved as one of the factors responsible for salt tolerance in Nebraska cultivar, application of foliar treatment with ascorbic acid could stimulate all the above mentioned parameters under normal and salt salinity stress. The two cultivars used have different mechanism to adapt to salt stress. Based on the responses and the ability of the kidney bean cultivars to cope with salinity stress, cultivar Nebraska could be recorded as more salt tolerance than Paulista.

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